Somatic embryogenesis and peroxidase activity of desiccation tolerant mature somatic embryos of loblolly pine

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Abstract: White, translucent, glossy mucilaginous callus was initiated from the mature zygotic embryos explants on callus induction medium with 2,4-D, BA, and kinetin in the 3-9th week of culture. This type of callus induction occurred at a lower frequency with either α-naphthaleneacetic acid (NAA) or IBA (both 8 mg/L). White, translucent, glossy mucilaginous callus was embryogenic and mainly developed from the cotyledons of the mature zygotic embryo. Somatic embryos were formed on differentiation medium. Desiccation tolerance can be induced by culturing somatic embryos of loblolly pine (*Pinus taeda* L.) on medium supplemented with 50 μm abscisic acid (ABA) and/or 8.5% polyethylene glycol (PEG_{6000}). Scanning electron microscopy of desiccated somatic embryos showed that the size and external morphology of the desiccation tolerant somatic embryos recovered to the pre-desiccation state within 24-36 h, whereas the sensitive somatic embryos did not recover and remained shriveled, after the desiccated somatic embryos had been rehydrated. Peroxidase activity of desiccated somatic embryos increased sharply after 3 days of desiccation treatment, and desiccation tolerant somatic embryos had higher peroxidase activity compared to sensitive somatic embryos. Higher peroxidase activity of desiccation tolerant somatic embryos was possibly advantage of catalyzing the reduction of H_2O_2 which was produced by drought stress, and protecting somatic embryos from oxidative damage.

Key words: *Pinus taeda* L.; Somatic embryogenesis; Desiccation tolerance ; Peroxidase activity

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Introduction

Somatic embryogenesis has been regarded as the in vitro system of choice with the potential for eventual mass propagation of superior and genetically engineering forest tree genotypes in both coniferous and hardwood species (Gupta and Durzan 1991; Attree and Fowke 1993, Zimmerman 1993; Park *et al.* 1998). Somatic embryogenesis in conifer was first described relatively recently by three independent groups: Hakman *et al.* (1985) and Chalupa (1985) described somatic embryogenesis from cultured zygotic embryos of Norway spruce, while Nagmani & Bonga (1985) reported the induction of haploid European larch (*Larix decidua* Mill.) embryo from megagametophytic tissue. In just a few years, mature conifer somatic embryos had been produced, of a quality with compares very favourable with mature zygotic embryos. Somatic embryogenesis of loblolly pine has been induced by Gupta et al. 1987, Becwar *et al.* 1990, and Li *et al.* 1998, and is considered to be the most promising method for large-scale propagation of loblolly pine and it offers the potential with automated system to produce large numbers of synthetic seeds (Attree *et al.* 1993; Becwar *et al.* 1995; Gupta *et al.* 1993; Handley *et al.* 1995).

Desiccation (partial drying) can improve the germination and plant regeneration response of somatic embryos in conifers (Attree *et al.* 1993). The successful induction of desiccation tolerance in somatic embryos has been reported for some angiosperm and conifer species (Attree *et al.* 1993, Florin *et al.* 1993; Bewley 1979; Brown *et al.* 1993; Iida *et al.* 1992; Seneratna *et al.* 1990). Partial drying has been described for Skita spruce (Robertson *et al.* 1991), red spruce (Harry & Thorpe 1991), black spruce (Tremblay & Tremblay 1991), white spruce (Kong and Yeuang 1992), and Norway spruce (Bozhkov *et al.* 1992). Robertson *et al.* (1990) first observed that partial drying of interior spruce somatic embryos at high relative humidity (RH>95%) increased germination frequency, decreased germination times, and improved the synchrony of root and shoot elongation compared to untreated somatic embryos. Of various chemicals and physical stresses have been applied to induce desiccation tolerance, abscisic acid (ABA) and polyethylene glycol (PEG) have been frequently employed (Attree *et al.* 1993; Florin *et al.* 1993; Zeevart *et al.* 1988). ABA and PEG influence the accumulation of specific mRNAs, storage reserves, storage lipid triacylglycerol (TAG) in conifer somatic embryos and zygotic embryos (Attree *et al.* 1992), of which some may be involved in seed protection. In some plant species, late embryogenesis abundant (LEA) proteins, whose accumulation increase dramatically at the beginning of zygotic embryo desiccation and which are regulated by ABA, are considered to be involved in protecting tissue from desiccation (Dure *et al* 1993). Leal

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and Misra (1993) reported that late developmental stage of white spruce zygotic embryos accumulated high levels of LEA transcripts. However, little is known about precise mechanism by which ABA and PEG induce desiccation tolerance in conifer somatic embryos. Peroxidase catalyzes the reduction of H_2O_2 and protects embryos from oxidative damage. There is no report about peroxidase activity of desiccation tolerant somatic embryos in conifer. In this investigation, we report the first evidence of increased peroxidase activity in desiccation tolerant somatic embryos of loblolly pine.

Materials and Methods

Plant material

Mature cones of genotypes E-822, E-311, and E-440 of loblolly pine were collected from 25-30 year old trees in the morning hours in October, 1996, from Yingde Seed Orchard at Yingde, Guangdong Province, and Shaoyang Seed Orchard at Shaoyang, Hunan Province, China. All cones were stored in plastic bags at 4 °C for 2 - 18 months. Seeds were disinfected by immersion in 70% v/v ethanol for 30 s and in 0.1% mercuric chloride for 20 min, followed by 4-5 rinses in sterile distilled water. Mature zygotic embryos were aseptically removed from the seeds and placed horizontally on a solidified callus induction medium in 100 mL Erlenmeyer flasks (40 mL medium in 100 mL flask, 5-8 explants per flask).

Callus and somatic embryo Induction

Callus and somatic embryo induction were carried out as described previously (Tang *et al.* 1998a). Mature zygotic embryos were cultured on callus induction medium consisted of TE (Tang *et al.* 1998) supplemented with 8 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 4 mg/l 6-benzylaminopurine (BA), and 4 mg/L Kinetin (KT). Callus was formed on cotyledons, hypocotyls, and radicles of mature zygotic embryos after 9 weeks on induction medium. Then callus was subcultured on the same medium with lowered 1/5 auxin and cytokinin concentrations. After 9 weeks, white, translucent, glossy mucilaginous calluses containing embryogenic suspensor masses (ESM) and immature somatic embryos was selected for proliferation on the medium consisted of TE medium supplemented with 2 mg/L 2,4-D, 0.5 mg/L BA, 0.5 mg/L indole-butyric acid(IBA), and 0.5 mg/L Kinetin. Then the influences of different concentrations of ABA, PEG_{6000}, and activated charcoal were tested. Each treatment was comprised of 150-200 explants and the experiment was repeated thrice. In the callus induction and proliferation media, the concentrations of sucrose, casein hydrolysate, glutamine, myo-inositol, and agar were 30 g/L, 500 mg/L, 500 mg/L, 500 mg/L, and 7 g/L, respectively. The pH was adjusted to 5.8 with 1N KOH or 0.5 N HCl prior to autoclaving at 121°C for 18 min. Callus induction took place in the darkness at 23°C. Data were analyzed with Analysis of Variance, and mean comparison was made with the Least Significant Difference test at 5% level of probability.

ABA and PEG treatment, desiccation, and rehydration

After 9 weeks of culture, mature somatic embryos were treated with ABA and PEG_{6000} to induce desiccation tolerance. The culture medium was replaced by TE supplemented with 50 μM ABA and/or 8.5% PEG_{6000}. As a control desiccation-sensitive preparation, the medium was replaced by TE without ABA and PEG_{6000}. After 7 days of incubation in dark at 25°C, embryos were selected by sieving through a 200 μm nylon mesh and then washed with sterile deionized water. These consisted of cotyledon-stage embryos. They were transferred to a sterile filter paper, which was moistened with a few drops of sterile deionized water, in a 60 mm x 15 mm plastic Petri dish. Somatic embryo desiccation was performed according to the method of Takahata *et al.* (1993). Embryos in a Petri dish were dried through a series of desiccators in which the relative humidity (RH) was kept constant using a saturated solution of K_2SO_4 (RH 87%), Na_2CO_3 (RH 80%), NaCl (RH 70%), NH_4NO_3 (RH 61%), $Ca(NO_3)_2 \cdot 4H_2O$ (RH 50%) or $K_2CO_3 \cdot 1.5H_2O$ (RH 40%). They were transferred daily from a desiccator at a higher RH to one at a lower RH. After the desiccation treatment, the filter paper bearing the embryos was transferred to TE agar (0.7%)-solidified medium containing 2% sucrose and incubated at 25°C under a 16-h photoperiod with light provided by cool white fluorescent bulbs (75 $\mu mol \cdot m^{-2} \cdot s^{-1}$).

Scanning electron microscopy

Embryogenic calluses were prepared for scanning electron microscopy according to Fowke *et al.* (1994) with some modification. Tissues were fixed overnight in 4% glutaraldehyde and 100 mM phosphate buffer (pH 7.0), washed one time in 100 mM phosphate buffer (pH 7.0) for 30 min, followed by dehydration in successive ethanol alcohol solution of 85%, 95%, and 100%, each repeated twice for 5 min. Specimens were dried in a critical-point-drier with CO_2 for 2 h, mounted on Cu stubs and gold-coated. The samples were examined and photographed in HITACHI S-800 scanning electron microscope.

Peroxidase activity

The enzyme activity was assayed according to the procedure reported by Kim and Yoo (1996), 0.1 g FW somatic embryos (30-50 somatic embryos) was homogenized in 1 mL phosphate buffer (pH 6.0, 0.1M) for 5 min. Homogenates were then centrifuged at 12 000 rpm for 5 min at 4°C and the supernatant was used for peroxidase activity assay. Peroxidase activity was determined at 30°C with a spectrophotometer (470 nm) following the formation of tetraguiaiacol in a 3-mL reaction mixture containing 1 mL of 0.1 M photophate buffer (pH 6.0), 1 mL of 15 mM 2-methoxyphenol (guaiacol); 1 mL of 3 mM H_2O_2; and 5 μl of enzyme extract. One unit of peroxidase activity (U)

represents the amount of enzyme catalyzing the oxidation of 1 mol of guaiacol in 1 min. Data were analyzed by an Analysis of Variance, and mean comparisons were made with the Least Significant Difference test at 5% level of probability.

Results and Discussion

Callus induction and somatic embryos maturation. Calli were formed from the mature zygotic embryos explants on media containing auxin and cytokinin in the 3-9th week of culture. As the calli started to proliferate, four types could be distinguished: white, translucent, glossy mucilaginous, light yellowish, loose, glossy globular, light green globular, and light brown globular. White, translucent, glossy mucilaginous callus was initiated from 16.7%, 19.8%, and 11.7% of the E-822, E-311, and E-440 mature zygotic embryos, respectively, on TE callus induction medium with 2,4-D, BA, and kinetin (Fig. 1). This type of callus induction occurred at a lower frequency with either α-naphthaleneacetic acid (NAA) or IBA (both 8 mg/l). White, translucent, glossy mucilaginous callus developed from the cotyledons of the mature zygotic embryo, light yellowish, loose, glossy globular, light green globular, and light brown globular calli formed from the hypocotyl as well as from the radicle of the explants. White, translucent, glossy mucilaginous callus was embryogenic, and was selectively proliferated on solid callus proliferation medium with 2 mg/L 2,4-D, 0.5 mg/L BA, 0.5 mg/L indole-butyric acid (IBA), and 0.5 mg/L kinetin or liquid callus proliferation medium supplemented with the same plant growth regulators as solid medium.

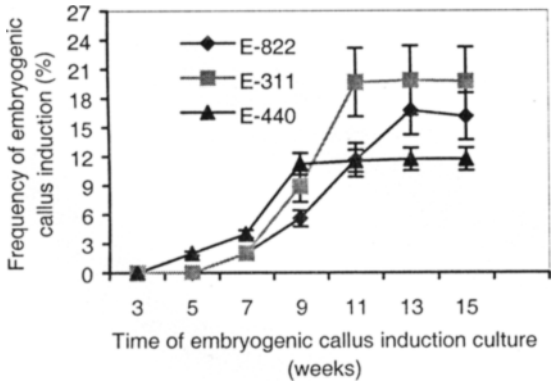

Fig. 1 Genotypes and the time of callus induction on the induction frequency of embryogenic callus cultures derived from mature zygotic embryos of loblolly pine

Each treatment was replicated three times, and each replicate consisted of 30–50 somatic embryos. Values represent the means ± S.D.

In order to encourage the maturation of loblolly pine somatic embryos, embryogenic tissue cultures containing embryogenic suspensor masses, immature somatic embryos, and globular somatic embryos must be transferred from an environment that promotes cleavage polyembryogenesis to one containing ABA and ideally raised osmotic concentration. ABA, PEG, and activated charcoal are important for the maturation of loblolly pine somatic embryos. ABA prevents the developing embryos from germinating precociously; PEG encourages the maturation of somatic embryos by regulating the osmotic pressure. Activated charcoal absorbs harmful compounds and hormones in tissues and encourages the maturation of somatic embryos (Attree *et al.* 1993; Gupta *et al.* 1993; Tautorus *et al.* 1991). However, the optimal ABA, PEG, and activated charcoal concentration required to promote maturation of loblolly pine somatic embryos needs to be determined. The highest frequency of somatic embryos (both precotyledonary somatic embryos and cotyledonary somatic embryos per gram cultures with the period of 12 weeks can be obtained when ABA, PEG_{6000}, and activated charcoal concentration are 4mg/L, 75g/L, and 5g/L, respectively (Fig. 2).

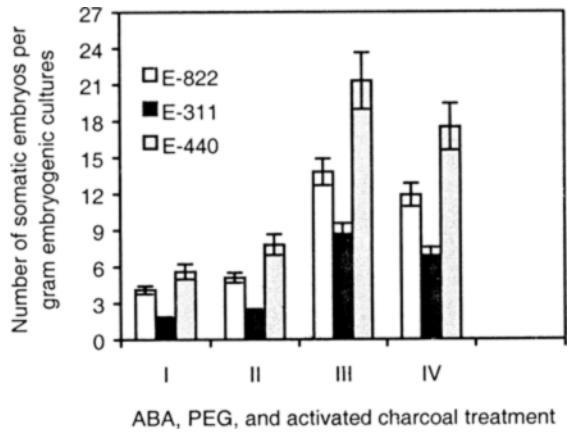

Fig. 2 Influence of ABA, PEG_{6000}, and activated charcoal on the number of somatic embryos per gram embryogenic callus cultures derived from mature zygotic embryos of loblolly pine.

I: 2 mg/L ABA, 20 g/L PEG_{6000}, and 2 g/L activated charcoal; II: 3 mg/L ABA, 50 g/L PEG_{6000}, and 3 g/L activated charcoal; II: 4mg/L ABA, 75 g/L PEG_{6000}, and 5 g/L activated charcoal; IV: 5mg/L ABA, 100 g/L PEG_{6000}, and 7 g/L activated charcoal. Each treatment was replicated three times, and each replicate consisted of 30–50 somatic embryos. Values represent the means ± S.D.

Scanning electron microscopy of desiccarion tolerant somatic embryos. This investigation showed that globular somatic embryos may be obtained from embryogenic calluses cultured on callus proliferation medium, but cotyledonary somatic embryos can be produced only from embryogenic calluses cultured on medium supplemented with ABA and PEG_{6000} (Fig. 3a-d). No visual differences in morphological appearance were observed between the somatic embryos treated and untreated with ABA and PEG_{6000}, except that the addition of ABA and PEG_{6000} reduced embryo growth slightly. Scanning electron micro-

scopical observation of non-desiccation embryos revealed that their epidermal cells were arranged regularly and that their cell surface was smooth, similar to the appearance reported by Nitta *et al.* (1997) and Wakui *et al.* (1999). Scanning electron microscopy of desiccated somatic embryos revealed that the external morphology of the desiccation tolerant and sensitive embryos differed. The whole surface of the desiccation tolerant embryos showed that a mesh like structure due to wrinkling of the cells, whereas the surface of desiccation sensitive embryos appeared to be torn and less wrinkled than those of the desiccation tolerant embryos. A similar morphology was reported in desiccation tolerant somatic embryos of white spruce (Fowke *et al.* 1994) and Brassica napus L. (Wakui *et al.* 1999). After transfer to TE germination medium (Tang *et al.* 1998a), desiccation sensitive embryos did not show recovery of their morphology to that before desiccation, and reminded shriveled with an irregular cell surface after 7-10 days of rehydration (Fig 3e). Desiccation tolerant somatic embryos imbibed water rapidly and within 24-36 h had regained the same size and appearance as they had before desiccation. Their epidermal cells appeared turgid and were arranged regularly (Fig. 3f). Most of these somatic embryos develop roots within 7-10 days.

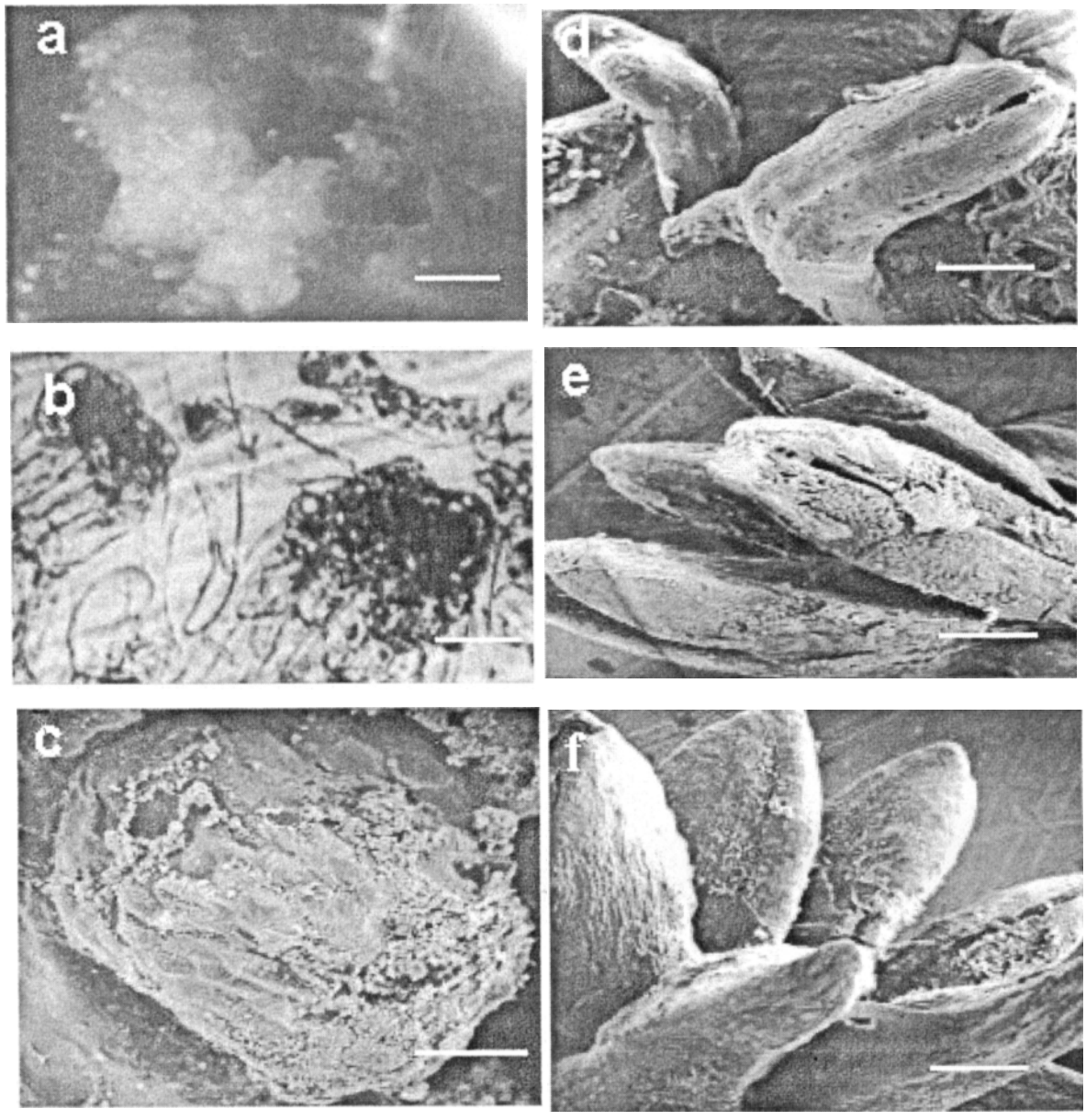

Fig. 3 Scanning electron microscopy of somatic embryogenesis in loblolly pine

a--Embryogenic callus (Bar = 1cm), b--Embryogenic suspensor mass (Bar = 0.1cm), c--Globular somatic embryo (Bar = 0.1mm), d--Cotyledonary somatic embryo (Bar = 0.2mm), e--Desiccation sensitive somatic embryo with an irregular cell surface (Bar = 0.3mm), f--Desiccation tolerant somatic embryo with regularly arranged epidermal cells (Bar = 0.3mm).

Peroxidase activity of desiccation tolerant somatic embryos. H_2O_2 levels increase in plant cells after exposure to many environmental stresses such as radiation, drought, wounding, extreme temperature, and pathgen attack (Baker and Orlandi 1995; Yahraus *et al.* 1995). Prolonged accumulation of toxic amount of H_2O_2 within the cell is eventually lethal. To avoid these deleterious effects, all organisms express peroxidase, which function as detoxifying enzymes by catalyzing the reduction of H_2O_2 (Creissen *et al.* 1994). This investigation results indicated that desiccation tolerant somatic embryos induced by ABA and/or PEG have higher peroxidase activity, which is advantage of the reduction of H_2O_2 produced by desiccation and the survival of the somatic embryos, as well as the maintenance of the tissue system during dehydration, protecting embryos from oxidative damage, compared to desiccation sensitive somatic embryos. Peroxidase activity of desiccation tolerant somatic embryos increased sharply after 3 days of desiccation treatments (Table1). It has been shown that ABA affects the stabilization of the cell membrance system (Gadea *et al.* 1999), induces the expression of late embryogenesis abundant (LEA) proteins for the stabilization of cell structure under dehydration stress (Dure 1993), and stimulates the accumulation of the storage lipid triacylglycerol (TAG) (Attree *et al.* 1992). This investigation suggested that ABA and/or PEG_{6000} also probably promoted the production of peroxidase of desiccation tolerant somatic embryos, which catalyzes the reduction of H_2O_2 and protect embryos from oxidative damage.

Table 1 Peroxidase activity of desiccation tolerant mature somatic embryos of loblolly pine under different relative humidity (RH).

Treatment	Peroxidase activity (U g-1 FW)*					
	87%**	80%	70%	61%	50%	40%
ABA	24.3±2.1b	25.9±2.9b	30.5±2.7b	42.1±4.2b	49.7±5.1b	50.6±4.7b
PEG_{6000}	21.5±1.6b	23.1±1.8b	27.2±1.8b	38.5±3.1b	45.2±3.5b	47.3±3.8b
$ABA+PEG_{6000}$	31.7±2.9a	33.8±3.4a	36.2±3.1a	47.9±3.9a	59.8±5.7a	61.2±7.6a
Control	11.8±1.3c	12.7±1.1c	13.5±1.4c	15.6±1.2c	16.2±1.1c	16.9±2.2c

*Peroxidase activity of somatic embryos was determined after 5-8 hrs of desiccation treatment. Each treatment was replicated three times, and each replicate consisted of 30 –50 somatic embryos (0.1 g FW). Values represent the means ± S.D. Means followed by the same letter are not significantly different at the 0.05 level of confidence. **Relative humidity (RH).

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